Use of biomass ash to reduce toxicity affecting soil bacterial community growth due to tetracycline antibiotics

V. Santás-Miguel[1], M.J. Fernández-Sanjurjo[2], A. Núñez-Delgado[2], E. Álvarez-Rodríguez[2], M. Díaz-Raviña[3], M. Arias-Estevez [1], D. Fernandez-Calviño[1]*

[1]Área de Edafoloxía e Química Agrícola. Facultade de Ciencias. Universidade de Vigo. As Lagoas 1, 32004 Ourense. Spain

[2]Departamento de Edafoloxía e Química Agrícola, Escola Politécnica Superior de Enxeñaría, Universidade de Santiago de Compostela. Campus univ. 27002 Lugo. Spain

[3]Departamento de Bioquímica del Suelo, Instituto de Investigaciones Agrobiológicas de Galicia (IIAG/CSIC). Santiago de Compostela. Spain

* Corresponding author E-mail: vsantas@uvigo.es (V. Santás Miguel)

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Abstract

Tetracycline antibiotics (TA) used in veterinary medicine reach terrestrial ecosystems mostly via the repeated applications of animal manures and slurries on agricultural soils, where they may cause toxic effects on bacterial communities. In the current work, we studied the efficacy of adding doses of 0, 6, 24 and 48 g kg^{-1} of biomass ash (BA) to four different soils to reduce potential negative effects of tetracycline antibiotics. Specifically, soil samples were polluted with different concentrations of tetracycline, oxytetracycline or chlortetracycline, and the bacterial community growth was estimated using the ^{3}H leucine incorporation technique. Soil amendment with BA increased soil pH (1.3-4.8 units), total carbon (0.7-5.8 g kg^{-1}) and Fe and Al oxides concentrations (0.25-3.98 g kg^{-1}), as well as bacterial activity (1-9 times compared to the control). In addition, BA amendment at high doses (24 or 48 g kg^{-1}) resulted in a similar toxicity decrease for the three antibiotics, but with variations among soils. The reductions in antibiotics toxicity were very variable, ranging between 5% and 100% (total recovery). In view of that, the spreading of BA could be interesting as management practice to reduce risks of soil pollution and subsequent toxicity on bacterial communities due to tetracycline antibiotics.

Keywords: Biomass ash; chlortetracycline; leucine incorporation; oxytetracycline; tetracycline

1. Introduction

Veterinary antibiotics are widely used for animal health, and even as animal growth promoters in countries where this parallel use is not banned (Anderson et al., 2005). These antibiotics reach terrestrial ecosystems via the repeated applications of

animal manures and slurries as amendments on agricultural soils (Pan and Chu, 2017). Once in the soil, veterinary antibiotics may cause harmful effects on non-target organisms, such as soil bacterial communities (Warman, 1980). The presence of antibiotics in the soil may cause a reduction in microbial biodiversity (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008), influence the growth and enzymatic activities of bacterial communities (Liu et al., 2009; Ma et al., 2016; Song et al., 2017; Santás-Miguel et al., 2020), and hence, ecological functions and functional stability (Zielezny et al., 2006; Demoling and Bååth, 2008; Pallecchi et al., 2008; Toth et al., 2011).

Tetracyclines are the antibiotics most used in the European Union, especially tetracycline, oxytetracycline and chlortetracycline **(**ESVAC, 2016), which motivated that previous studies have focused on the effects of tetracycline residues on soil microbial functions (Thiele-Bruhn, 2005; Yang et al., 2010; Liu et al., 2012; Ma et al., 2016; Song et al., 2017). It has been established that the main agents responsible for the sustainability of soils are microorganisms (Pulleman et al., 2012), since they are involved in nutrient cycles and renewal of organic matter (Thiele-Bruhn et al., 2012). Therefore, the correct growth of soil bacterial communities will help to maintain an optimal soil fertility and organic matter turnover. However, there are only few studies estimating the effects of tetracycline antibiotics on soil bacterial community growth (Rousk et al., 2008; Rousk et al., 2009a; Santás-Miguel et al., 2020**).** These studies have shown a marked toxicity due to tetracycline antibiotics, which was highly dependent on soil characteristics.

In order to prevent harmful effects of antibiotics accumulated into soils, the use of appropriate waste and/or by-products as soil amendments may be a low cost alternative aiding to decrease undesirable toxicity affecting soil bacterial communities. It has been previously demonstrated that soil amendment with certain by-products, such

as mussel-shell (Fernández-Pazos et al., 2013; Osorio-López et al., 2014; Otero et al., 2015; Romar-Gasalla et al., 2018), pyritic material (Fernández-Pazos et al., 2013; Osorio-López et al., 2014; Otero et al., 2015), pine bark (Romar-Gasalla et al., 2018), oak ash (Romar-Gasalla et al., 2018) and hemp waste (Romar-Gasalla et al., 2018) may reduce the availability of different types of pollutants, both anionic and cationic. However, even if is considered that the amendment of soils with by-products may stimulate or inhibit microbial communities, the potential effects of by-products on soil bacterial community growth has been poorly studied. Within the few researches previously carried out in this regard, Fernández-Calviño et al. (2015a) assessed the effect of different doses of crushed mussel shell applied in two Cu-polluted acid mine soils, showing long-term positive effects. In a subsequent study, Fernández-Calviño et al. (2018) measured bacterial and fungal growth in acid mine soils amended with pine bark, crushed mussel shell and a mixture of both, showing general positive effects on microbial growth. However, no previous studies have dealt with the effects of biomass ash amendment on bacterial community growth in soils.

Biomass ash (mainly derived from biomass energy plants and combustion boilers of variate sizes) is a very abundant waste material, which increases soil pH, as well as the contents of major nutrients, and decreases the availability of Al and some minor elements **(**Demeyer et al., 2001). Also, biomass ash may reduce the availability of different pollutants in soils (Núñez-Delgado et al., 2015; Núñez-Delgado et al., 2017; Romar-Gasalla et al., 2018). Specifically regarding effects on microbiota, Zimmermann and Frey (2002) studied the effect of wood ash amendment on soil microbial process, showing an increase in pH and a subsequent stimulation of microbial biomass, basal respiration and enzymatic activities.

In view of this background, in the current work we hypothesize that adding appropriate doses of biomass ash to soils, the bacterial community growth would be stimulated, and toxicity effects due to tetracycline antibiotics would decrease. Therefore, the main objective of this work is to assess the effect of biomass ash amendments on bacterial community growth in soils polluted with the tetracycline antibiotics tetracycline, oxytetracycline and chlortetracycline.

2. Material and Methods

2.1. Chemicals

Tetracycline hydrochloride (CAS. 64-75-5; ≥95% in purity), Oxytetracycline hydrochloride (CAS 2058-46-0; ≥95% in purity) and Chlortetracycline hydrochloride (CAS 64-72-2; ≥97% in purity), all three supplied by Sigma–Aldrich (Steinheim, Germany), were used for soil spiking.

2.2. Procurement of soil and biomass ash samples

Four soils devoted to cultivation of potatoes in rotation with cereal, located in Galicia (NW of Spain), previously analyzed by Conde-Cid et al. (2019a)**,** were selected for the current study. On each sampling site 10-20 sub-samples were taken in the soil surface horizon (0-20 cm) using an Edelman probe, and were subsequently mixed in one composite sample. At the laboratory, the soil samples were air dried, sieved by a 2 mm mesh light screen, homogenized and stored in polyethylene bottles. Soil pH was determined in water (soil ratio: 1:2.5), using a combined glass electrode. Total carbon and total nitrogen were determined by elemental analysis in a LECO CHN-1000 (LECO Corporation, St. Joseph, MI, USA). Exchangeable basic cations (Ca, Mg, Na and K) were extracted with 0.2 M NH_4Cl (Sumner and Miller, 1996), while exchangeable Al

was extracted with 1 M KCl (Bertsch and Bloom, 1996), and then determined by flame atomic absorption (Ca, Mg and Al) or emission spectroscopy (Na and K). The effective cation exchange capacity (eCEC) was estimated as the sum of the exchangeable basic cations and Al. Fe and Al oxides (Fe_o and Al_o) were extracted with 0.2 M ammonium oxalate-oxalic acid (Blakemore, 1978).

The main characteristics of these soils are shown in Table 1. Briefly, these soils present pH values (measured in water) between 4.6 and 5.1, organic carbon contents between 1.1 and 3.8%, and effective cation exchange capacity between 4.1 and 6.8 $cmol_c\ kg^{-1}$. Al and Fe oxides have values ranging between 855-5040 mg kg^{-1}, and between 1150-2585 mg kg^{-1}, respectively.

The biomass ash (BA) used was from a combustion boiler in Lugo (Spain). Previously, this by-product was analyzed by Romar-Gasalla et al. (2018). Briefly, it showed a pH in water of 11.3, a total carbon content of 11.7%, a total nitrogen content of 0.21%, and a C/N ratio of 55.5. It presented high contents of amorphous Al and Fe oxides (8323 and 4233 mg kg^{-1} respectively), and also relatively high levels of oxides of Ca and other elements (Table 1), due the combustion process.

2.3. Experimental Design

Four soils were amended with four doses of biomass ash (0, 6, 24 and 48 g kg^{-1}), resulting in 16 soil mixtures. In brief, 288 dry grams of each soil sample were weighed in four 500 mL polyethylene bottles (72 g per bottle) and mixed with different amounts of biomass ash (0, 0.43, 1.73, 3.46 g) in order to obtain the final concentrations of 0, 6, 24 and 48 g of biomass ash per kg^{-1} of soil. These concentrations were satisfactorily used in previous works testing the effects of different by-products on soils (Ramírez-Pérez et al., 2013; Fernández-Calviño et al., 2015a; Conde-Cid et al., 2020a; Conde-Cid

et al., 2020b). Later on, the resulting mixtures of dry soil and different concentrations of biomass ash were rewetted up to 60-80% of water-holding capacity, and the bottles sealed with lab-film (Parafilm) to avoid soil drying. Then, these mixtures were incubated during 30 days at 22 ºC in the dark.

After this incubation time, different amounts of tetracycline, oxytetracycline and chlortetracycline were dry added (via talc) separately (by triplicate) to the 16 soil mixtures in 50 mL polypropylene tubes, reaching 8 concentrations for each antibiotic and each mixture (0, 0.5, 2, 7.8, 31.3, 125, 500 and 2000 mg kg^{-1}). The bottles were sealed with Parafilm to avoid soil drying (thus maintaining water-holding capacity). The resulting microcosms (totalizing a number of 960, 1 g each one) were initially studied for 1 day, focusing on the assessment of the subsequent bacterial community growth, performed after extraction from soil samples, and estimated using the ^{3}H Leucine incorporation method (Bååth 1994; Bååth et al., 2001). Briefly, 1 g of soil (fresh weight) was mixed with 10 mL distilled water using a multivortex shaker for 3 min at maximum intensity, followed by low-speed centrifugation at 1000 *x g* for 10 min, to create a bacterial suspension in the supernatant. An aliquot (1 mL) of this suspension was transferred to 2 mL micro-centrifugation tubes. Then, 2 µL [^{3}H]Leu (3.7 MBq mL^{-1} and 0.574 TBq $mmol^{-1}$; Perkin Elmer, USA) were added with non-labeled Leu to each tube, resulting in 275 nM Leu in the bacterial suspensions. After incubation for 2 h at 22 °C, bacterial growth was stopped with 75 µL 100% trichloroacetic acid. Washing was performed as described by Bååth et al. (2001), and the amount of leucine incorporated was determined using scintillation liquid counting (Tri-Carb 2810 TR, Perkin Elmer, USA).

2.4. Data analysis

Data on bacterial growth obtained for each soil and each concentration of tetracycline, oxytetracycline or chlortetracycline were normalized (Santás-Miguel et al., 2020) respect to the control (samples without antibiotic) to be able to compare the inhibition curves of different soils and concentrations of biomass ash. The normalized data were represented as inhibition curves of relative bacterial growth as a function of antibiotic doses. From the inhibition curves, a toxicity index (log IC_{50}) was calculated to compare the effects of different doses of BA regarding the toxicity exerted by antibiotics on soil bacterial communities. The logarithm of the concentration that inhibits 50% of bacterial growth (log IC_{50}) was calculated using the following logistic model: $Y= c/[1+e^{b(a-x)}]$, where Y is Leu incorporation for each antibiotic concentration, x is the logarithm of the concentration of antibiotic added, a is the value of log IC_{50}, b is a parameter related to the slope of inhibition curves, and c is the bacterial growth without antibiotic addition.

3. Results and discussion

3.1. Biomass ash effect on general soil characteristics and bacterial community growth

In soils without BA (dose 0 g kg^{-1}) the values of soil pH varied between 4.6 and 5.1 (Table 1). However, soil pH changed significantly after biomass ash addition, following dose-response curves (Fig. 1). Thus, in soil samples amendment with 6 g kg^{-1} of biomass ash, pH increased between 1.3 and 2.4 units. Regarding soils amended with 24 g kg^{-1} and 48 g kg^{-1} of BA, pH values increases between 2.9 and 4.2 units, and between 3.6 and 4.8 units, respectively. Total carbon in soils without BA varied between 11 and 38 g kg^{-1} (Table 1), values in the range of those usually found in Galicia (Calvo de Anta et al., 2015). Carrying out a theoretical calculation, the addition of BA (which contains 11.7% of C, Table 1) to agricultural soils contributed to an increase of

193 total carbon amounting: 0.7 g kg^{-1} for a dose of 6 g kg^{-1} of BA; 2.9 g kg^{-1} for a dose of
194 24 g kg^{-1} of BA; and 5.8 g kg^{-1} for a dose of 48 g kg^{-1} of BA. The contents of Fe and Al
195 oxides in the soils studied varied between 1.15 and 2.59 g kg^{-1}, and between 0.86 and
196 5.04 g kg^{-1}, respectively (Fe_o and Al_o contents in Table 1). Carrying out the theoretical
197 calculation of Fe and Al oxides added to each soil by BA amendment, the results
198 indicated that the addition of 6 g kg^{-1} of BA to soils contributed to an increase of 0.25
199 and 0.50 g kg^{-1} in the contents of Fe and Al oxides; while the increase ranged between
200 1.01 and 1.99 g kg^{-1} for a BA dose of 24 g kg^{-1}; and reached up to between 2.02 and
201 3.98 g kg^{-1} for a dose of 48 g kg^{-1} of BA (Table 2).
202 The behavior regarding mean bacterial growth in response to soil amendment with
203 biomass ash (Fig. 2) was different for each soil. In soil 1 and 2 it was observed that the
204 effect on bacterial growth due to BA addition was low (increases between 1-1.9 times as
205 compared to control), while it was high for soils 3 and 4 (increases of up to 9.8 and 7.0
206 times as compared to the control). Previous studies also found increases in bacterial
207 community growth in response to soil amendment with various by-products, such as
208 crushed mussel shells **(**Fernández-Caviño et al., 2015a**)**, pine bark (Fernandez-Calviño
209 et al., 2018), and marble wastes or organic wastes (Zornoza et al., 2016). These
210 increases were generally attributed to both increases in soil pH and in total carbon, two
211 characteristics also increased by biomass ash amendments. In relation to soil pH, Rousk
212 et al. (2009b) found increases of 5-fold in bacterial community growth for a gradient of
213 soil pH going from 4 up to 8. Organic matter also shows high importance for bacterial
214 growth, and different authors found important increased in bacterial community growth
215 in mine soils amended with organic materials (Zornoza et al., 2016; Fernández-Calviño
216 et al., 2018).
217

3.2. Effect of biomass ash on the toxicity exerted by tetracycline antibiotics on bacterial community growth

Figure 3 shows the bacterial community growth inhibition curves as a function of the antibiotic dose, for the 3 antibiotics (tetracycline, oxytetracyline and chlortetracycline), as well as for the 4 soils studied and the 4 different BA doses added. Table 3 shows the estimated log IC_{50} values obtained by fitting the inhibition curves to the logistic model. Log IC_{50} values show that CTC was the most toxic antibiotic for bacterial community growth in absence of BA, while differences between OTC and TC are not clear. In the current study, adding BA to the soils, the toxicity exerted on bacterial community growth decreased for the three antibiotics (i.e. all three behave similarly). It was observed that, by increasing the biomass ash dose added to soils, the inhibition curves moved to the right with respect to the curve corresponding to the control (no BA added), and therefore the log IC_{50} values increased. However, the BA dose needed to reduce significantly the toxicity of the antibiotics was different for the various soils here studied. Thus, in soils with low organic carbon (1.07%), the toxicity of the antibiotics was considerably reduced using 24 g kg^{-1} of BA, with negligible inhibition of bacterial community growth being observed. Conversely, in the soils with higher organic carbon (3.1 to 3.8%), a clearly higher BA dose (48 g kg^{-1}) was needed to decrease toxicity. These results suggest that the effectiveness of BA amendment for decreasing antibiotics toxicity on soil bacterial communities will be greater in soils with lower organic carbon than in those with higher organic carbon content.

The fact of achieving reductions of TC, OTC and CTC toxicity on bacterial community growth in presence of biomass ash may be due to different reasons. One reason may be that at high pH the tetracycline antibiotics became less toxic, since (according to Gesche et al., 1991) tetracyclines antimicrobial action is optimum at pH 6.0. Also, Gesche et al.

(2001) showed that oxytetracycline has a more effective antimicrobial action when it is on a substrate adjusted at acidic pH (around 6.0) than in substrates at basic pH (around 8.0). The addition of BA to the soils used in the current study significantly increased the soil pH from acid to basic values, with magnitude dependent on BA dose. It is relevant that the effect of pH on the activity of the tetracycline antibiotics is due to their amphoteric character. Specifically, depending on soil pH, tetracycline antibiotics can be in cationic, zwitterionic or anionic form, and increases in pH due to biomass ash amendment may cause that the charge of tetracycline antibiotics go from cationic to zwitterionic or even anionic forms (Table 4). Furthermore, it must be noted that the species of these antibiotics determine their passage inside the bacteria, and therefore its toxicity. Tetracycline antibiotics diffuse through the plasmatic membrane of bacteria as positively charged cations (Yamaguchi et al., 1991), specifically through porin channels (Chopra et al., 1992**),** due the presence of Donnan equilibrium through the outer membrane (Stock et al., 1977). However, the diffusion of anionic tetracycline antibiotics species is hindered (Stock et al., 1977).

Another mechanism explaining reductions in the toxicity of tetracycline antibiotics in presence of biomass ash is the increase of antibiotics adsorption onto soils due to the amendment. In fact, biomass ash has a relevant capacity for retention of tetracycline antibiotics (Conde-Cid et al., 2019b). The increase of total carbon due to biomass ash amendment enhances the surface area in soils, facilitating antibiotics adsorption by soils, making them less bio-available and decreasing their toxicity on microbial communities. This hypothesis is consistent with results found by different authors who observed that organic carbon plays an important role in tetracyclines adsorption onto soils (Zhao et al., 2011a; Zhao et al., 2012; Fernández-Calviño et al., 2015a). In addition, Fernández-Calviño et al. (2015b) studied sorption kinetics for

tetracycline, oxytetracycline and chlortetracycline in two acid soils, with results showing high affinity for all three tetracyclines on both soils, although adsorption intensity was greater in the case of the soil containing more organic matter, clay and Al and Fe oxides. In the current work, the biomass ash amendment increased total soil carbon content, but also increased Al and Fe oxides (Fe_o and Al_o), which may also contribute to tetracyclines adsorption onto soils. Besides, this by-product contains Ca, Fe (and other) oxides generated in the combustion process, and they may be related to sorption of anionic species through polyvalent cations, which can act as a cation bridge, linking organic matter and anionic species of the tetracycline antibiotics. In this sense, MacKay and Canterbury (2005) studied the adsorption of OTC on humic acids amended with metals in cationic form (Ca, Al and Fe), and observed that the addition of Al increased adsorption of OTC due to formation of ternary complexes between functional groups of the organic matter with negative charges, and zwitterion and anionic species of OTC. In addition, changes in soil pH can also affect to tetracycline antibiotics adsorption on soils, as shown by Figueroa and Mackay (2004), who investigated the adsorption of oxytetracycline on goethite and hematite and found that sorption increased with pH, reaching maximum values at about pH 8. Therefore, the effect of increasing soil pH due to the biomass ash amendment may also contribute to reduce tetracycline antibiotics toxicity on bacterial community growth.

4. Conclusions

Soil amendment with biomass ash contributed to increase pH values, total carbon and Al and Fe oxides contents, increasing also the bacterial activity in the amended soils. Moreover, it can be a good alternative to reduce antibiotics toxicity exerted on bacterial community growth. However, the effectiveness of this practice will

293 be different depending on soil type. Specifically, in soils with low total carbon content
294 the biomass ash doses needed for achieving clear reductions in antibiotics toxicity are
295 lower than in soils with high total carbon contents. These results could be of relevance
296 to program correct management practices for soils affected or in risk to be affected by
297 pollution due to tetracycline antibiotics.
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Table 1. General characteristic of the soils and biomass ash studied. Average values (n=3) with coefficients of variation always <5%

	Soil 1	Soil 2	Soil 3	Soil 4	Biomass ash
C (%)	1.07	3.75	3.10	3.39	11.65
N_T (%)	0.09	0.34	0.25	0.31	0.21
pH_w	4.80	5.05	4.63	4.74	11.31
Ca_e ($cmol_{(+)}\ kg^{-1}$)	1.53	3.67	1.51	2.24	95.03
Mg_e ($cmol_{(+)}\ kg^{-1}$)	0.41	0.71	0.52	0.64	3.26
Na_e ($cmol_{(+)}\ kg^{-1}$)	0.25	0.12	0.21	0.35	12.17
K_e ($cmol_{(+)}\ kg^{-1}$)	1.27	1.22	0.93	1.00	250.7
Al_e ($cmol_{(+)}\ kg^{-1}$)	0.61	1.10	2.16	1.68	0.07
eCEC ($cmol_{(+)}\ kg^{-1}$)	4.08	6.82	5.33	5.92	361.2
Al_o ($mg\ kg^{-1}$)	855	4335	4950	5040	8323
Fe_o ($mg\ kg^{-1}$)	1150	2430	1390	2585	4233

pH_W is pH measured in water; C is total organic carbon; N_T is total nitrogen; eCEC is the effective cation exchange capacity ($cmol_c kg^{-1}$). X_e is exchangeable concentration of the element ($cmol_{(+)}\ kg^{-1}$); Al_o, Fe_o: extracted with ammonium oxalate ($mg\ kg^{-1}$).

Table 2. Final concentrations of Fe and Al oxides in soils for each dose of biomass ash added to the soils studied. Average values (n=3) with coefficients of variation always <5%

	Biomass ash dose							
	0 g kg^{-1}		6 g kg^{-1}		24 g kg^{-1}		48 g kg^{-1}	
Soil	**Al_o**	**Fe_o**	**Al_o**	**Fe_o**	**Al_o**	**Fe_o**	**Al_o**	**Fe_o**
Soil 1	0.86	1.15	1.35	1.40	2.85	2.16	4.84	3.17
Soil 2	4.34	2.43	4.83	2.68	6.33	3.44	8.32	4.45
Soil 3	4.95	1.39	5.45	1.64	6.94	2.40	8.93	3.41
Soil 4	5.04	2.59	5.54	2.84	7.03	3.60	9.02	4.61

Al_o, Fe_o: extracted with ammonium oxalate (mg kg^{-1})

Table 3. Values of logarithm IC_{50} (estimated values ± error) and R^2 from fits to logistic model for the 3 antibiotics and 4 soils studied as a function of different biomass ash (BA) doses added.

		Soil 1		Soil 2		Soil 3		Soil 4	
Antibiotic	**BA dose**	**Log IC_{50}**	**R^2**	**Log IC_{50}**	**R^2**	**Log IC_{50}**	**R^2**	**Log IC_{50}**	**R^2**
TC	**0 g kg^{-1}**	2.26±0.09	0.975	2.71±0.06	0.985	2.51±0.05	0.989	2.68±0.08	0.973
	6 g kg^{-1}	1.84±0.09	0.992	3.14±0.01	0.998	2.49±0.05	0.991	2.83±0.12	0.957
	24 g kg^{-1}	-	-	2.80±0.1	0.963	3.35±0.03	0.992	2.85±0.18	0.941
	48 g kg^{-1}	-	-	5.84±2.91	0.541	3.58±0.62	0.814	4.64±1.12	0.735
OTC	**0 g kg^{-1}**	2.11±0.13	0.975	1.90±0.07	0.995	2.70±0.10	0.948	2.46±0.16	0.934
	6 g kg^{-1}	2.77±0.10	0.971	2.96±0.21	0.906	1.80±0.05	0.997	2.49±0.07	0.983
	24 g kg^{-1}	-	-	2.87±0.10	0.970	2.84±0.11	0.921	3.09±0.10	0.921
	48 g kg^{-1}	-	-	4.75±1.53	0.695	3.64±0.13	0.929	3.35±0.46	0.818
CTC	**0 g kg^{-1}**	1.82±0.12	0.985	2.00±0.08	0.982	2.15±0.08	0.965	2.42±0.03	0.996
	6 g kg^{-1}	1.79±0.09	0.985	1.88±0.18	0.953	1.63±0.10	0.990	1.81±0.16	0.973
	24 g kg^{-1}	-	-	2.17±0.12	0.987	3.08±0.07	0.953	3.99±0.26	0.943
	48 g kg^{-1}	-	-	3.36±0.18	0.280	-	-	2.43±0.45	0.926

TC: tetracycline; OTC: oxytetracycline; CTC: chlortetracycline

Table 4. Percentage of each antibiotic species for different pHs measured in the amended soils. Average values (n=3) with coefficients of variation always <5%

			TC				OTC				CTC			
Soil	**BA dose (g kg^{-1})**	**pH**	**TC^{+1}**	**TC^{0}**	**TC^{-1}**	**TC^{-2}**	**OTC^{+1}**	**OTC^{0}**	**OTC^{-1}**	**OTC^{-2}**	**CTC^{+1}**	**CTC^{0}**	**CTC^{-1}**	**CTC^{-2}**
1	**0**	5.0	2.04	97.79	0.16	0.00	1.63	98.03	0.34	0.00	1.95	97.89	0.16	0.0
	6	7.4	0.01	70.44	29.36	0.19	0.00	52.74	45.93	1.32	0.01	71.39	28.42	0.18
	24	9.1	0.00	3.47	72.52	24.01	0.00	0.93	40.51	58.56	0.00	3.67	73.19	23.1
	48	9.7	0.00	0.52	42.91	56.57	0.00	0.09	14.79	85.12	0.00	0.55	44.02	55.4
2	**0**	5.1	1.63	98.17	0.21	0.00	1.30	98.28	0.43	0.00	1.56	98.25	0.20	0.0
	6	6.3	0.10	96.70	3.20	0.00	0.08	93.44	6.46	0.01	0.10	96.84	3.06	0.0
	24	7.9	0.00	42.63	56.20	1.17	0.00	24.97	68.76	6.27	0.00	43.78	55.12	1.10
	48	8.6	0.00	12.05	79.61	8.34	0.00	4.74	65.38	29.88	0.00	12.59	79.46	7.95
3	**0**	4.6	4.98	94.95	0.06	0.00	4.00	95.87	0.13	0.00	4.77	95.17	0.06	0.0
	6	6.3	0.10	96.70	3.20	0.00	0.08	93.44	6.46	0.01	0.10	96.84	3.06	0.0
	24	8.1	0.00	31.66	66.15	2.19	0.00	16.68	72.80	10.52	0.00	32.70	65.24	2.06
	48	8.9	0.00	5.90	77.83	16.26	0.00	1.86	51.33	46.81	0.00	6.21	78.19	15.6
4	**0**	5.1	1.63	98.17	0.21	0.00	1.30	98.28	0.43	0.00	1.56	98.25	0.20	0.0
	6	6.7	0.04	92.28	7.68	0.01	0.02	81.93	17.92	0.13	0.04	92.60	7.36	0.01
	24	8.2	0.00	26.74	70.33	2.93	0.00	13.34	73.32	13.34	0.00	27.69	69.54	2.77
	48	8.9	0.00	5.90	77.83	16.26	0.00	1.86	51.33	46.81	0.00	6.21	78.19	15.6

TC: tetracycline; OTC: oxytetracycline; CTC: chlortetracycline. The superscript (TC^{X}.OTC^{X} and CTC^{X} is the valence of the antibiotic.

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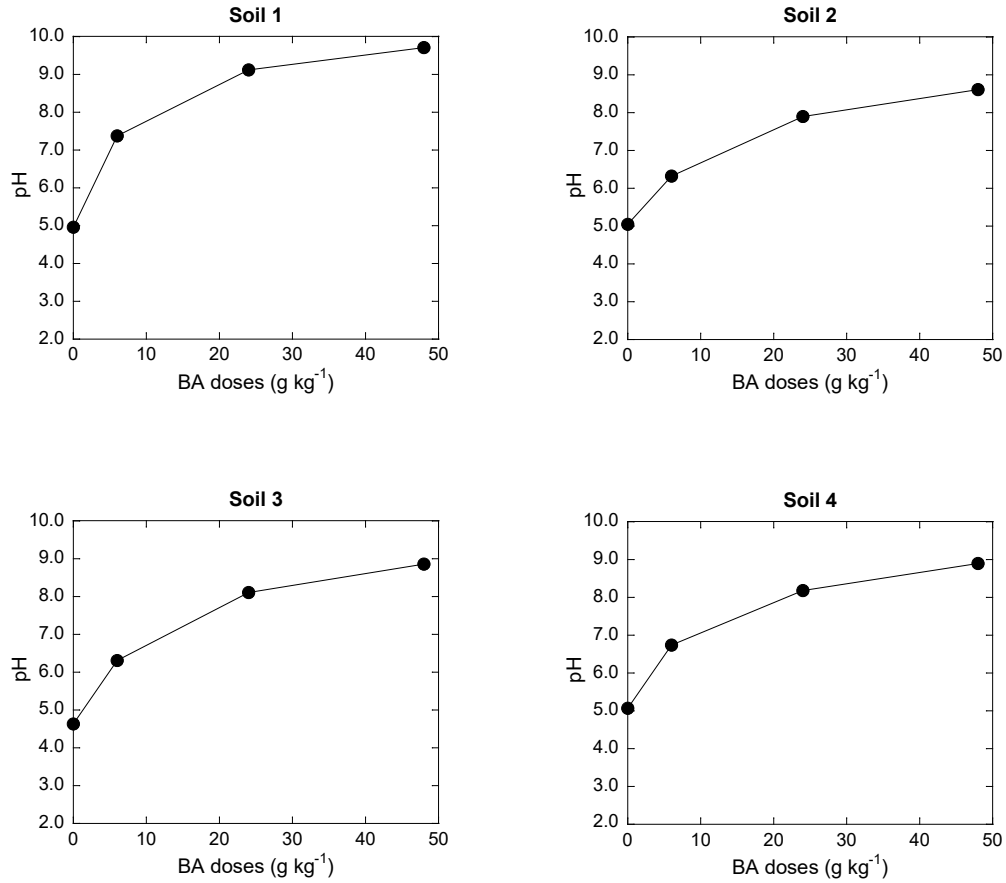

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512 **Fig. 1**. pH variation as a function of Biomass Ash (BA) doses added to soils (0, 6, 12 and 48 g kg^{-1}).

513 Average values (n=3) with coefficients of variation always <5%

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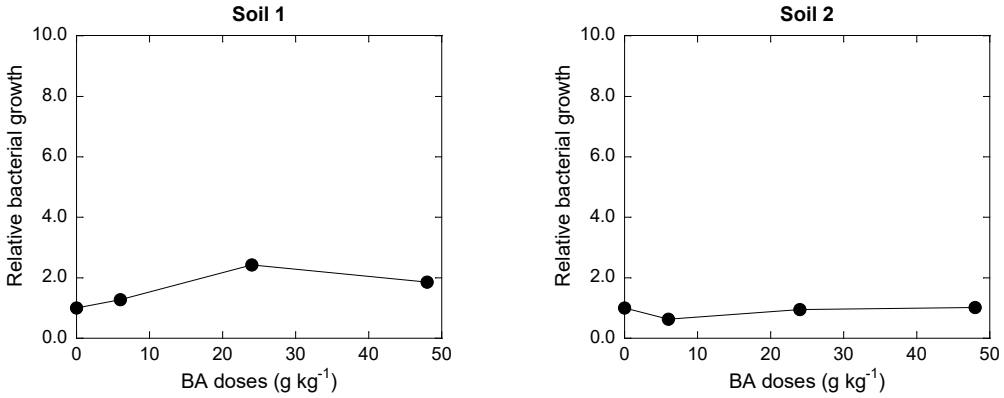

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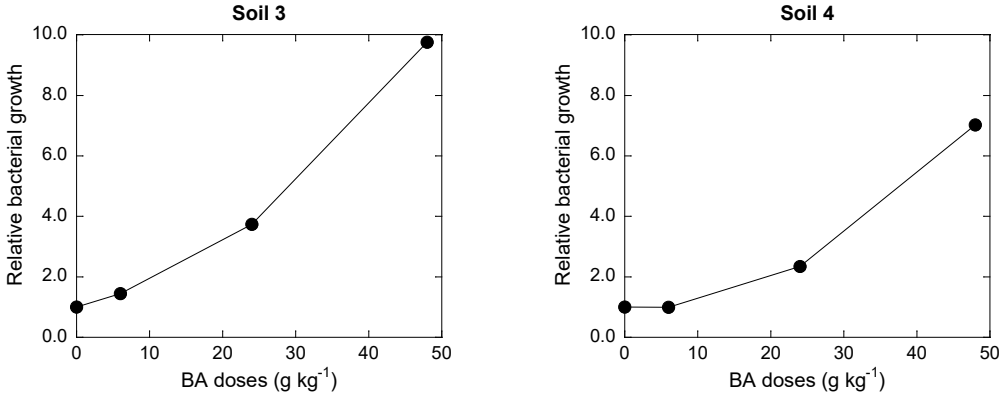

521 **Fig. 2.** Relative bacterial growth in control samples (without antibiotics) as a function of different doses
522 of Biomass Ash added (0, 6, 24 and 48 g kg^{-1}). Average values (n=3) with coefficients of variation always
523 <5%

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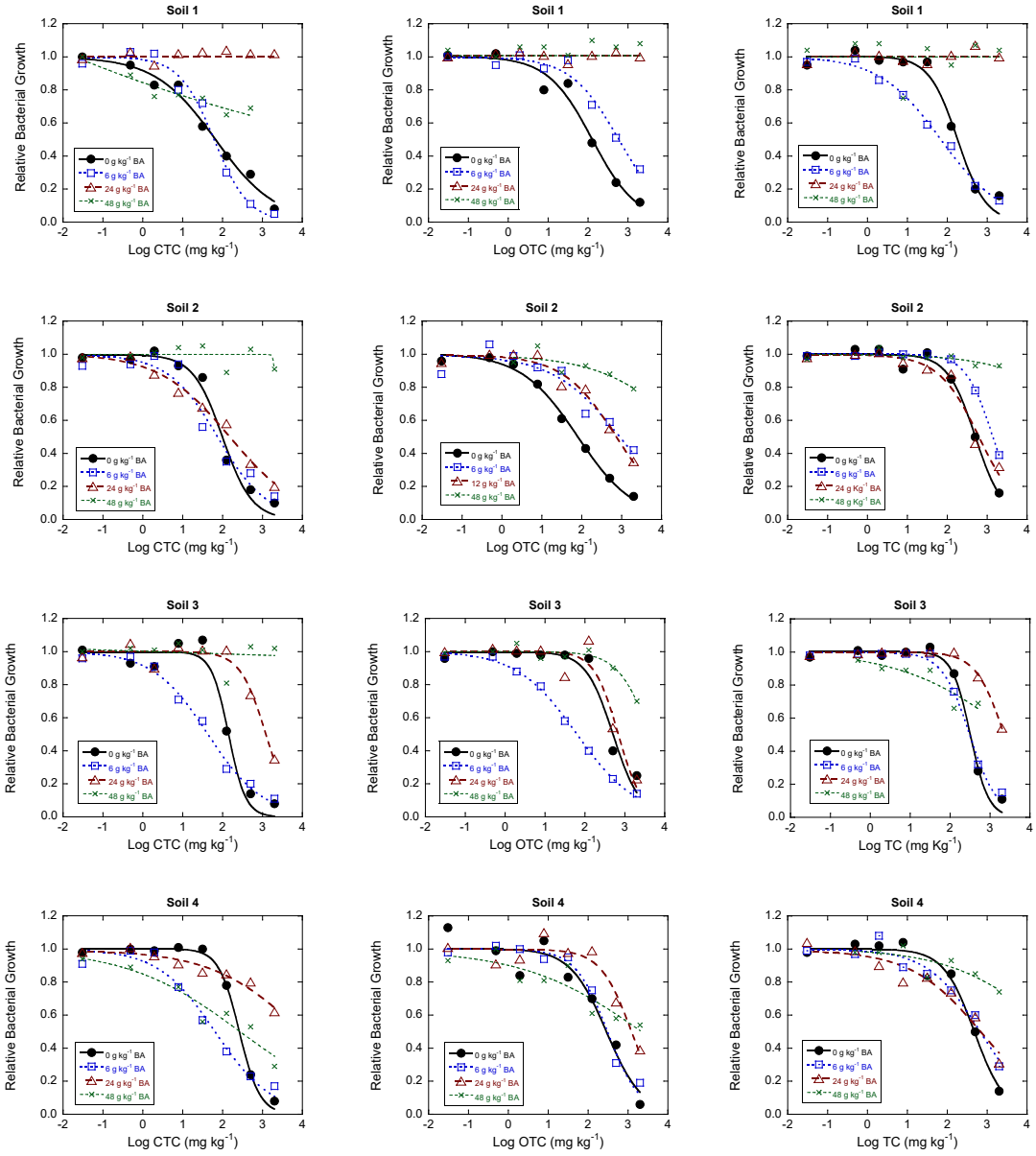

531 **Fig. 3.** Bacterial community growth dose-response curves for 4 soils, 3 antibiotics [Tetracycline (TC),
532 oxytetracycline (OTC) and chlortetracycline (CTC)], and 4 biomass ash (BA) doses. Average values
533 (n=3) with coefficients of variation always <5%.